International Immunopharmacology 28 (2015) 344–353

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

DNMT1 activates the canonical Wnt signaling in rheumatoid arthritis model rats via a crucial functional crosstalk between miR-152 and the DNMT1, MeCP2

Cheng-gui Miao [a,b], Dan Qin [a], Chuan-lai Du [a], Hua Ye [a], Wei-jing Shi [a], You-yi Xiong [a], Xiao-lin Zhang [a], Hao Yu [a], Jin-feng Dou [a], Shi-tang Ma [a], Mei-song Qin [a], Han-zhen Liu [a], Yan-xi Fang [a], Guo-liang Zhou [a], Jian-zhong Chen [a], Xu He [b], Cheng Huang [b], Yan Huang [b], Bing Zhang [c], Tong-wen Song [d], Jun Li [b,*]

[a] School of Food and Drug, Anhui Science and Technology University, Fengyang 233100, China
[b] School of Pharmacy, Anhui Medical University, Hefei 230032, China
[c] First Affiliated Hospital, Anhui Medical University, Hefei 230032, China
[d] First Affiliated Hospital, Bengbu Medical College, Bengbu 233000, China

ARTICLE INFO

Article history:
Received 30 March 2015
Received in revised form 20 May 2015
Accepted 7 June 2015
Available online 18 June 2015

Keywords:
Rheumatoid arthritis
DNMT1
Wnt signaling
miR-152
MeCP2

ABSTRACT

In previous study, we identified that microRNA (miR)-152 expression was down-regulated in RA model rats, and overexpression of miR-152 inhibited the canonical Wnt signaling through the DNA methyltransferase (DNMT1) inhibition. However, the exact molecular mechanisms of DNMT1 were unclear. In this work, we investigate whether DNMT1 affects the pathogenesis of RA model rats and targets the miR-152 promoter. The effects of DNMT1 on the canonical Wnt signaling, the pathogenesis of RA model rats and the SFRP1 expression were detected by the real time qPCR, Western blotting, ELISA, MTT and viable cell number assay. The interaction between miR-152 and DNMT1, methyl CpG binding protein 2 (MeCP2) was investigated by real time qPCR and chromatin immunoprecipitation (ChIP). Our results revealed that increased DNMT1 activated the canonical Wnt signaling could not only by targeting SFRP4 may also by SFRP1 in RA model rats. Furthermore, treatment of DNMT1 inhibitor, 5-aza-2′-deoxycytidine (5-azadC), or knockdown of DNMT1, or knockdown of MeCP2 led to increased miR-152 expression by reversion of its promoter hypermethylation, DNMT1 and MeCP2 binding to the CpG islands of miR-152 promoter. Interestingly, it is proved a synergistic inhibition effect of DNMT1 and MeCP2 in this process. Moreover, overexpression of miR-152 could inhibit DNMT1 expression and result in a decrease of DNMT1 and MeCP2 binding to miR-152 promoter, and inhibition of miR-152 expression would reverse it. These observations demonstrate a crucial functional crosstalk between miR-152 and the DNMT1, MeCP2 by a double-negative circuit involved in the pathogenesis of RA model rats.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, systematic and autoimmune disease that can severely affect synovial membranes and multiple joint structures [1,2]. In some cases, it also can be associated with extra-articular manifestations, mainly involving heart, lung and renal functions [3]. Apart from clinical and radiological features, RA related autoantibodies are also important markers for diagnosis, especially in the early phase of disease. In particular, anti-CCP antibodies are considered as highly specific diagnostic markers for severe erosive RA, and their early identification allows the early diagnosis and treatment of RA patients, who experience better outcomes [4]. Emerging evidence has supported an important role for microRNAs (miRNAs) in RA, and dysregulated miRNA expression seems to contribute to the underling pathogenesis of the disease [5]. Aberrant expression of several miRNAs has already been detected in RA, in different cell types, in different specimens, and these miRNAs can regulate specific signaling pathways, thus leading to the inflammatory milieu taking place in RA [6]. For example, Dong et al. found that decreased expression of miR-21 correlated with the imbalance of Th17 and Treg cells in patients with RA. Thus, miR-21 may serve as a novel regulator in the homoeostasis of T-cell differentiation, and provides a new therapeutic target for the treatment of RA patients [7].

Research shows that fibroblast-like synoviocytes (FLS) participate in the synovial hyperplasia, inflammatory cytokine secretion, cartilage erosion, and suggests that FLS display a crucial role in RA pathogenesis [8]. The Wnt gene is originally identified as a segment polarity gene in

Abbreviations: RA, rheumatoid arthritis; miRNA, microRNA; DNMT1, DNA methyltransferase 1; MeCP2, methyl CpG binding protein 2; ChIP, chromatin immunoprecipitation; 5-azadC, 5-aza-2′-deoxycytidine; SFRP, secreted frizzled-related protein; FLS, fibroblast-like synoviocytes; 3′UTR, 3′ untranslated regions; PBMC, peripheral blood mononuclear cells; NC, negative control.

* Corresponding author at: School of Pharmacy, Anhui Medical University, Mei Shan Road, Hefei, Anhui Province 230032, China. Tel./fax: +86 551 5161001.
E-mail address: lijunamu@126.com (J. Li).

http://dx.doi.org/10.1016/j.intimp.2015.06.013
1567-5769/

Drosophila melanogaster, and the Wnt signaling pathway displays an important role in bone formation, limb development, organogenesis and tumorigenesis [9]. Evidence has suggested the important role of the Wnt signaling pathway in the pathogenesis of RA [10]. In previous study, our group identified that increased methyl-CpG-binding protein 2 (MeCP2) reduced the secreted frizzled-related protein 4 (SFRP4) expression in FLS in RA model rats and the DNA methyltransferase (DNMT) inhibitor 5-Aza-2′-deoxycytidine (5-azadC) up-regulated the SFRP4 expression, indicating that DNMT has an important role in the differential expression of SFRP4 [11].

In view of the directly down-regulation of miR-152 on DNMT1 expression by targeting the 3′ untranslated regions (3'UTR) of its transcript in nickel sulfide-transformed human bronchial epithelial cells [12], we investigated whether miR-152 is aberrantly expressed and targets DNMT1 during the RA development. Based on our previous finding, miR-152 was specifically down-regulated in RA model rats, whereas overexpression of miR-152 directly resulted in a marked reduction of DNMT1 expression. Furthermore, increased miR-152 indirectly up-regulated the SFRP4 expression, a negative regulator of Wnt signaling pathway, through the DNMT1 inhibition. In addition, overexpressed miR-152 could suppress the canonical Wnt pathway activation and result in a significant decrease of FLS proliferation. Thus, miR-152 may provide molecular mechanisms for the activation of Wnt signaling in RA by targeting DNMT1. However, the exact molecular mechanisms of DNMT1 in RA pathogenesis were unclear. In this work, we used the RA model rats as animal model of RA pathology study, and investigate whether DNMT1 affects the canonical Wnt signaling, the pathogenesis of RA model rats and targets the CpG islands of miR-152 promoter, discussing the possibility of DNMT1 as a therapeutic target for RA patients.

2. Materials and methods

2.1. Preparation of RA model rats

Adult male Sprague–Dawley (180–200 g) rats were used to prepare the RA model rats. Model rats were prepared with complete Freund's adjuvant by paw injection, and controls were prepared with PBS. After model preparation, the synovium was isolated for further analysis. Animals were provided by the Experimental Animal Center of Anhui Medical University. All the animal experiments in this work followed the protocols approved by the Animal Care and Use Committee of Anhui Medical University.

2.2. Cell culture

FLS were cultured from the synovium which was isolated from RA model rats and controls. All cells were cultured at 37 °C in 5% CO_2 in cell culture flasks using high glucose DMEM medium supplemented with the Penicillin–Streptomycin Solution (Beyotime, China) and the 15% (*v*/*v*) heat-inactivated fetal bovine serum (FBS) (Hyclone, USA).

2.3. Real time qPCR

To perform qPCR, the total RNA was isolated by TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Reverse transcription was performed with 1 μg total RNA by the RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA) following the standard protocol. Production amplification was done by the QuantiFast® SYBR® Green PCR kit (Qiagene, Germany) on an ABI Prizm 7500 PCR machine. The following primers were used in this study: β-catenin forward, CTTACGGCAATCAGGAAAGC and β-catenin reverse, ACAGACAG CACCTTC AGCACT; ccnd1 forward, GCCCTCCGTTTCTTACTTCAA and ccnd1 reverse, CT CTTCGCACTTCTGCTCCTC; C-myc forward, ATTTCTAT CACCAGCAACAGCA and C-myc reverse, ATTTCTATCACCAGCAACAGCA; MMP3 forward, TGATGA ACGATGGACAGATGA and MMP3 reverse, AGCATTGGCTGAGTGAAAGAG; fibronectin forward, GACACTATGCGG GTCACTTG and fibronectin reverse, CCCA GGCAGGAGATTTGTTA; SFRP1 forward, GTGGGCTACAAGAAGATGGTG and SFRP1 reverse, GAAGAGCGAGCAGAGGAAGAC; β-actin forward, CCCATCTA TGAGGG TTACGC and β-actin reverse, TTTAATGTCACGCACGATTTC. Quantitative amplification reaction was carried out at 95 °C for 10 min, following 40 cycles at 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s.

2.4. Western blotting

Synovium or cells were lysed by the cell lysis buffer for Western and IP kit (Beyotime, China). Protein concentration was measured by the Enhanced BCA Protein Assay Kit (Beyotime, China) following the manufacturer's instructions. For Western blotting, 20 mg protein was separated by SDS-PAGE and blotted onto PVDF membranes (Millipore Corp, USA). After blocking, blot strips were incubated for 6 h in antibody buffer (Beyotime, China) including primary antibodies. The following primary antibodies were used in western blotting: rabbit polyclonal anti-β-catenin was purchased from Cell Signaling Technology (Beverly, MA, USA), rabbit polyclonal anti-fibronectin was purchased from Santa Cruz (Santa Cruz, USA), goat polyclonal anti-MMP3 from Santa Cruz (Santa Cruz, USA), rabbit polyclonal anti-SFRP1 was purchased from Santa Cruz (Santa Cruz, USA), Mouse monoclonal antibody directed against β-actin from Santa Cruz (Santa Cruz, USA). The anti-mouse, anti-rabbit and anti-goat antibodies (Boster, China) conjugated with horseradish peroxidase were used as secondary antibodies. After extensive washing 3 times with PBST, the protein blots were detected by the ECL-chemiluminescent kit (ECL-plus, Thermo Scientific).

2.5. Cell proliferation assay

Transfected FLS were cultured in 96-well plates for 24 h. After the cell concentration reached 0.5–1 × 10^5/mL, the FLS were cultured for another 4 h with 20 μL MTT (5 mg/mL) (Sigma, USA). Then the FLS with MTT reagent were resuspended in 150 μL DMSO (Sigma, USA) to dissolve the formazan, and the optical density values of the solution were determined at 490 nm using a Thermomax microplate reader (bio-tek EL, USA). Viable cell number assay was counted each day for 4 days in FLS transfected with DNMT1 siRNA and negative control or DNMT1 vectors and negative control. The data were expressed as mean ± standard error of the mean.

2.6. ELISA

The concentration of IL-6 and IL-8 in FLS supernatant was measured by the quantification ELISA kits (Yuanye Bio-Technology Co., Ltd, China) according to the manufacturer's instruction. Optical density values were detected at 450 nm. Three replicate wells were quantified for every sample, all experiments were performed in triplicate.

2.7. ChIP

ChIP assay was performed using EZ ChIP Assay Kit (Upstate Biotechnology) following the manufacturer's instruction with minor modifications. Briefly, when FLS were grown to cover the 95% bottom of cell culture flasks (1 × 10^6 cells), protein was cross-linked to DNA by addition of formaldehyde directly to the culture medium to a final concentration of 1% for 10 min at room temperature. The cross-linking reaction was quenched by 0.125 M glycine solution adding for 5 min at room temperature. Then the medium was removed, and FLS were collected and suspended in sodium dodecyl sulfate lysis buffer containing 1 × protease inhibitor cocktail (Upstate Biotechnology). FLS were sonicated to yield fragments of 500 bp average size, and the sonicated samples were precleared with 60 μL of salmon sperm DNA/protein G agarose beads for 1 h at 4 °C with agitation. The soluble chromatin fraction was collected, and 1% of the supernatant was applied for input normalization. Five microliters of rat IgG, anti-RNA polymerase II (Upstate